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13. ABSTRACT (Maximum 200 words)

Results obtained during this contract identify the metabolic pathway for cGMP as a potential site of action of tetanus toxin. Preliminary studies have revealed that guanylate cyclase activity is not inhibited in intoxicated PC12 cells. Although we can not rigorously rule out a possible role for this enzyme in toxin action, all of the evidence reported here is consistent with the view that the degradation of cGMP is stimulated in toxin-treated cells. The phosphodiesterase inhibitors, IBMX and zaprinast, were effective in reversing the effects of tetanus toxin on both the inhibition of evoked cGMP accumulation and ACh release in a similar manner. IBMX, a wide spectrum, rather low affinity, phosphodiesterase inhibitor, partially restored cGMP levels and ACh release. Zaprinast has been reported to be specific for cGMP-degrading phosphodiesterases in a number of diverse tissues.

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13. Abstract (continued)

Results reported here reveal that this agent was very effective in elevating cGMP levels in control PC12 cells as well. This compound completely restored the stimulus-evoked cGMP response and ACh release after it was applied for 15 min to intoxicated cells. While it is possible that the hydrophobic agents, 8Br-cGMP and zaprinast, act through nonspecific mechanisms, the observation that the effects of tetanus can be reversed by these two distinctly different chemicals that share the common property of elevating cGMP levels in PC12 cells strongly argues against nonspecific mechanisms.

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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TABLE OF CONTENTS

TETANUS TOXIN - SIGNIFICANCE

Tetanus toxin, the enterotoxin produced by the bacterium *Clostridium tetani*, is one of the most potent neurotoxins known (minimal lethal dose of toxin in mice, 2 ng/kg body weight). This toxin shares many common properties with botulinum toxin, a group of neurotoxic substances also produced by Clostridial bacteria. These toxins have a common bacterial origin, similar molecular structures, and most likely the same mechanism of toxic action at the subcellular level (for reviews see Simpson, 1986; Habermann and Dreyer, 1986). The most striking feature in the action of these toxins, beside their potency, is that their site of action is the presynaptic nerve terminal where they inhibit neurosecretion without causing cell death. Thus studies on the mechanism of action of the Clostridial neurotoxins should not only provide methods to prevent or reverse the toxic sequelae of these lethal bacterial infections but will also provide valuable insight into the molecular events that underlie the neurosecretion process.

It has been recognized for some time that the effects of tetanus toxin are specific for neural tissues, which is due, in part, to the specific recognition of neural tissues by the toxin. Evidence gathered by the principal investigator and others supported the notion that the specific high affinity receptors for tetanus toxin were polysialo-gangliosides (Rogers and Snyder, 1981; Walton *et al.*1988; Staub *et al.*1986). However, there has also been evidence to suggest that protein plays some role in the high affinity binding site (Pierce *et al.*1986; Critchley *et al.*1986). Thus the precise nature of the tetanus toxin receptor remains to be characterized and more work is needed to assess the physiological importance of gangliosides as binding molecules.

It is now clear that the initial binding step of the Clostridial toxins is nontoxic. In fact tetanus is like several other microbial toxins that participate in a complex multi-step intoxication pathway (Middlebrook and Dorland, 1984). Various steps in the pathway have been studied in

neural tissues (Schmitt *et al.*1981; Bergey *et al.*1983; Collingridge *et al.*1980). Recently, the principal investigator, utilizing an established preparation of tetanus toxin-sensitive PC12 cells, clearly identified a rapid, temperature-dependent internalization step following toxin binding to the surface (Sandberg *et al.*1989). Further, there was a clear lag phase which followed internalization, revealing that other intracellular events, such as processing of the toxin and expression of some enzymatic activity, are obligatory events in the pathway (Sandberg *et al.*1989). At present there is no information on the toxin processing events, the compartments in which they occur, or on the enzymatic activity or substrates of tetanus toxin. The PC12 cell system developed by the applicant represents an ideal system in which to address these important issues.

An emphasis of recent research has been to identify the putaitive enzymatic activity of the Clostridial neurotoxins. By analogy with other toxins, such as diphtheria and cholera, a number of investigations have focused on a potential ADP-ribosyltransferase activity for tetanus and botulinum. Although certain forms of botulinum toxin (C1 and D) can ADP-ribosylate a low molecular weight protein in adrenal medulla, there is evidence that this reaction is not related to inhibition of neurosecretion (Adam-Vizi *et al.*1989). In the case of tetanus toxin, there is no evidence for ADP-ribosyltransferase or any other enzymatic activity for that matter (Simpson, 1986). The lack of information on the precise target or substrate for these toxins has made progress difficult in this area. One of the important goals of this project has been to identify these substrates and develop probes so that the underlying enzymatic activity of tetanus toxin can be discovered.

The molecular mechanism that underlies the inhibitory effects of the Clostridial neurotoxins are not known. There is some evidence that the Ca^{2+} sensitivity of the release process is decreased (Mellanby and Green, 1981). Although there is one report that tetanus toxin blocks Ca^{2+} channels in cultured neuronal cells, substantial evidence indicates that neither tetanus nor

botulinum toxin act on Ca^{2+} channels (Dreyer *et al.*1983; Simpson, 1986). cGMP was implicated in the toxic action with the report that Clostridial neurotoxins inhibited guanylate cyclase in neural tissues (Smith and Middlebrook, 19). The principal investigator has obtained substantial evidence to implicate cGMP metabolism with the action of tetanus toxin in PC12 cells (see below and Sandberg et al., 1989). There has also been an interesting recent report that tetanus toxin decreases protein kinase C activity in macrophages and neural tissues from infected mice (Ho and Klempner, 1988). Consistent with this result are recent reports in which protein kinase C stimulated secretion in permeabilized pituitary cells and PC12 cells (Naor *et al.*1989; Ahnert-Hilger and Gratzl, 1988). The precise relation between these different observations is unclear. Accordingly, one of the important goals of this proposal is to identify an underlying relationship, or lack thereof, between protein kinase C, cGMP and tetanus toxin in neurosecretion.

RESULTS FROM THE PRINCIPAL INVESTIGATOR'S LABORATORY DURING THIS CONTRACT

Development of a model system to study the mechanism of action of tetanus toxin -- A major initial goal of this reserch program was to develop a model cultured cell system of neural origin that would serve as a valuable tool to study the underlying molecular mechanisms of action of Clostridial toxins. We chose to examine the rat pheochromocytoma cell line, PC12, since it has one of the most highly developed neurotransmitter release systems of any cultured cell line (Greene and Tischler, 1982). Thus initial studies were directed toward determining if these cells contained complex gangliosides, tri- and tetrasialoganliosides, and bound tetanus toxin with high affinity. As shown in Figure 1, PC12 cells did bind tetanus toxin with high affinity, with Kd's in the range of 1 to 2 nM.

During the past several years the PI has made significant progress toward identifying the site of action of tetanus toxin in PC12 cells (Sandberg *et al.*1989; Sandberg *et al.*1989; Evans and Rogers, 1988). We have found that when PC12 cells are depolarized or stimulated there are increases in cGMP which peak within 1 min. When the cells are treated with tetanus toxin, there is a dose-dependent blockade of both ACh release and increase stimulus-evoked cGMP accumulation (Fig. 1).

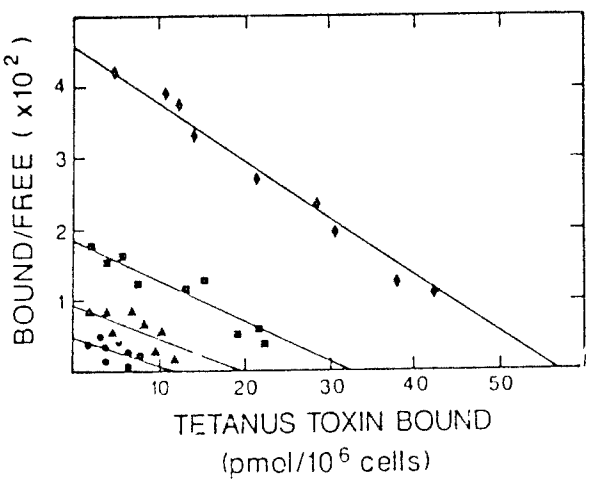
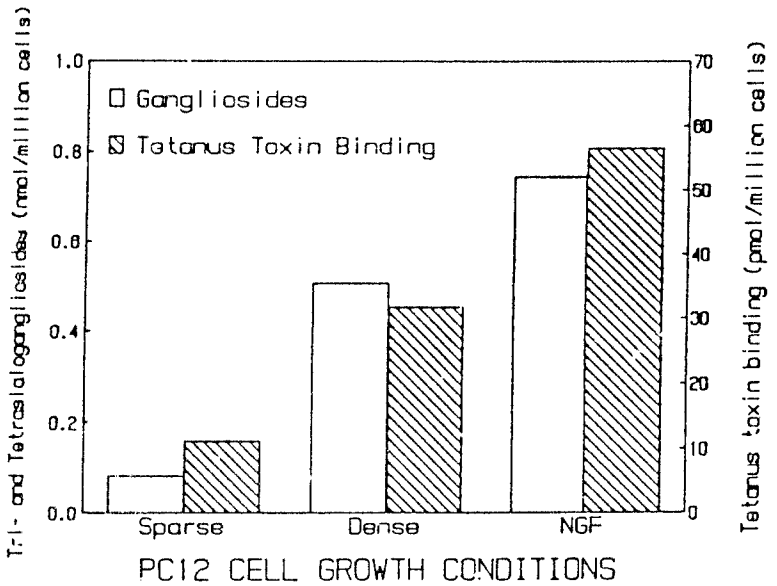


Figure 1 Scatchard analysis of tetanus toxin binding to PC12 cell membranes. ^{125}I-tetanus toxin (from .1 to 100 nM) was incubated with membranes prepared from PC12 cells (250 ng protein). Shown are the scatchard plots from experiments with membranes prepared from cells grown under different growth conditions: (●), sparse; (▲), dexamethasone; (◉), desnse cells; (◆), NGF-treated cells.

An important insight that we derived from these results was that PC12 cells express high affinity receptors for tetanus toxin and that nerve growth factor (NGF) resulted in a 6-fold increase in binding sites wsitrhout altering the Kd of the receptors. When we compared the level of tetanus toxin receptors with the expresion of complex gangliosides in these cels, we found an ecellent correlation as shown in Figure 2.

Figure 2. Comparison of PC12 trisialoganglioside expression and tetanus toxin binding. Trisialogangliosides were quantitiated by extraction and resolution on TLC plates. The levels of tetanus toxin receptors and gangliosdies are expressed per million cells.

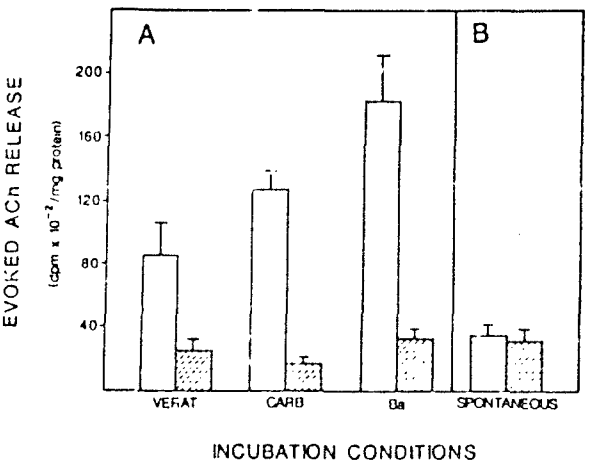


These results provide circumstantial evidence that the tetanus toxin binding and complex ganglioside levels are related, although no proof is provdied from such studies. These results are discussed in detail in a publication by the PI (Ahnert-Hilger *et al.*1985). Taken together these studies did provide important initial evidence that PC12 cells may indeed be an appropriate system to study the action of tetanus toxin.

At this stage the crusial question was whether or not the PC12 cells were sensitive to the

effects of tetanus toxin. Thus we examined the effetcs of tetanus toxin on ACh release from these culture cells. As shown in Figure 3, when tetasnus toxin-pretreated cells were depolarized by a vareity of secretagogues, there was an 80% inhibition of ACh release from these cells.

Figure 3. Effects of tetanus toxin on stimuluus-evoked ACh release from PC12 cells. [^{3}H]ACh release from NGF-treated PC12 cells was assayed over a 2 min interval following application of secretagogues; 200 μM veratridine (VERAT), 1 mM carbachol (CARB), or 2 mM $BaCl_2$. Spontaeous release was measured in parallel experiments over the samo time interval in the absence of stimulus. Data shows the levels of ACh release in control (open bars) and toxin-treated cells (hatched bars).



The inhibitory effects of tetanus toxin were dose- and time dependent. As shown in Figure 4, whent PC12 cells were incubated with increasing doses of tetanus toxin, maximal inhibition was observed at 2.5 nM, with half maximal efects observed at 0.5nM. These data are consistent with the binding data of Figure 1.

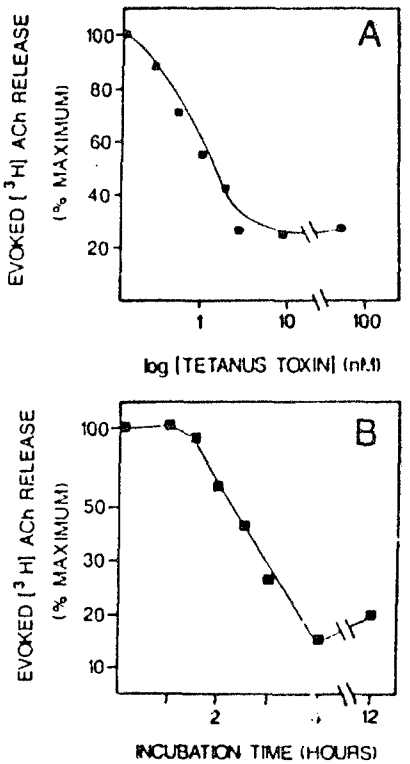


Figure 4. Dose response and time course for tetanus toxin inhibition of ACh release from PC12 cells. [^{3}H]ACh release from NGF-treated PC12 cells was assayed over a 2 min interval. In Panel A, cells were incubated for 3 hr with increasing concentrations of tetanus toxin. The results are copmared to cultures that were not incubated with toxin. In panel B cells were incubated with 10 nM tetanus toxin and the level of ACh release was assayed at various time intervals as shown.

In the time course studies there was a characteristic 1.5 hr lag phase before the onset of the inhibitory effects. Following this lag phase there is a rapid onst of the toixc effects (Figure 4B). It was possible that this lag phase was due to a slow penetratrion of the toxin into the PC12 cell. In order to examine this possibility, we utilized a protease protection assay that were have previously developed with N18 RE105 cells (Staub *et al.*1986). As shown in Figure 5, at 37°C there is a rapid internalization of tetanus toxin while at low temperature, the toxin remains on the surface of the cell.

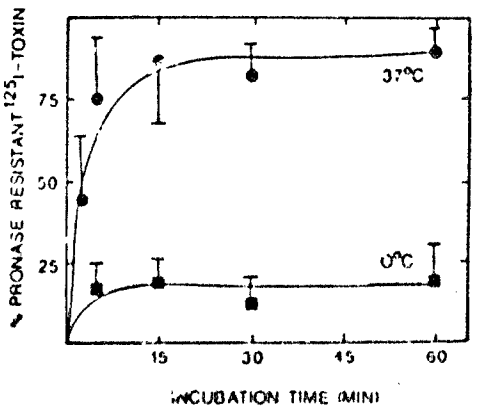


Figure 5. Tetanus toxin internalization into PC12 cells. Cells were incubated with 0.2 nM [125I]-tetanus toxin for 30 min at 0°C. At the end of this incubation cells were rinsed and then incubated at either 37°C (•) or 0°C (■). At various times as indicated the cultures were incubated with pronse to remove surface-bound toxin. The appearance of a protease-resistant fraction is indicative of toxin internalization.

These data reveal that the toxin is rapidly internalized yet the onset of the inhinibtory effects are more delayed. These data are consistent with a mechanism which includes several obligatory intracellular steps, such as processing and activation, prior to the expression of inhibition. Thus these results are consistent with physiologialc studies that suggest that the toxic effects of tetanus toxin develop only after several essential steps occur in the intracellular compartment (Bergey *et al.*1983; Collingridge *et al.*1980; Dreyer *et al.*1983).

One of the interesting properties of PC12 cells is that they can be differentiated in a number of different ways (Greene and Tischler, 1982). Thus we examined the effects of cell differentiation on the sensitivity to tetanus toxin. The cells were cultured under conditions known to stimulate distinct forms of differentiation: nondifferentiated, low density for 7 days (SPARSE); glucocorticoid treatment, in the presence of dexamethasone for 14 days (DEX); NGF for 14 days (NGF); autodifferentiated, high density for 7 days (DENSE). As shown in Fig 6A, the culturing conditions had a marked effect on the sensitivity of the cells to tetanus toxin.

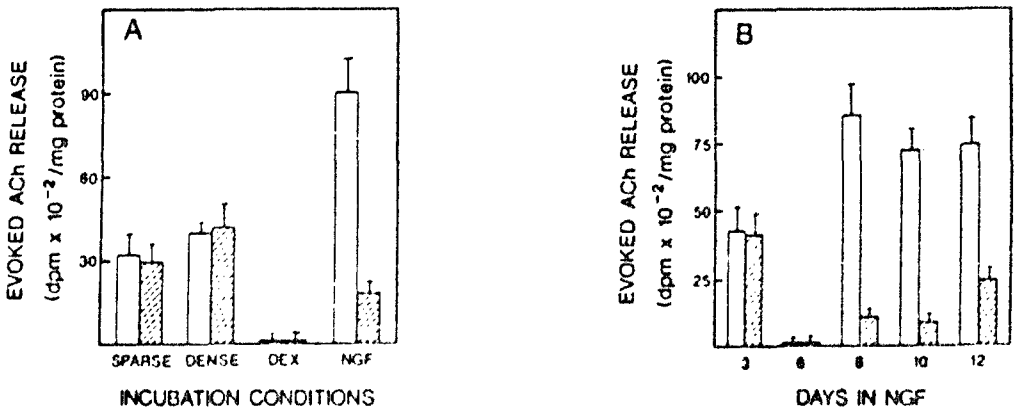


Figure 6. Effect of tetanus toxin on veratridine-evoked acetylcholine release from PC12 cells grown under various differentiation conditions. Veratridine-evoked [^{3}H]ACh release was measured as described in Fig. 1. In Panel A evoked [^{3}H]ACh release was measured in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM, 16-18 h incubation at 37°C) from PC12 cells grown under the following conditions: 14 days at 5 x 10^4 cells/10 cm^2, in the presence of 1 x 10^{-6} M dexamethasone (DEX), 14 days at 5 x 10^4 cells/10 cm^2, in the presence of 100 ng/ml nerve growth factor (NGF); 7 days, at high density (5 x 10^5 cells/ 10 cm^2) (Dense), or at low density (5 x 10^4 cells/ 10 cm^2) (Sparse). The results are the means of 2-3 experiments each performed in sextuplet ± s.e.m. Panel B shows the effect of tetanus toxin on veratridine-evoked acetylcholine release from PC12 cells as a function of days in NGF. Evoked [^{3}H]ACh release was measured as a function of culture days in NGF (100 ng/ml) in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM, 16-18 h incubation at 37°C)

In these experiments [^{3}H]ACh release from NGF treated cells was inhibited by 81% whereas cells grown under any of the other conditions were insensitive to tetanus toxin. There was a larger evoked release of [^{3}H]ACh from NGF-treated cultures which can be explained, in part, by the 8-fold higher levels of choline acetyltransferase (CAT) expressed in these cells. CAT levels (in pmol ACh/min/mg protein) were: sparse, 140 ± 12; DEX-treated, 156 ± 16; dense, 802 ± 69; and NGF-treated, 988 ± 86. It is noteworthy that densely grown cells, which differentiate to express CAT at elevated levels, and do show a significant evoked release of [^{3}H]ACh, are completely insensitive to the toxin. Closer examination of the development of sensitivity of NGF-treated cultures revealed that PC12 cells become sensitive to tetanus toxin only after culturing in NGF for 3 days or longer (Fig. 6B). Day 6 cells were particularly poor at releasing ACh. The characteristics of [^{3}H]ACh release from day 3 cells were similar to that observed in densely grown

cells (Fig. 6A). This may reflect the fact that day-3 cells may be more similar to dense cells since day-0 cells were subcultured from confluent flasks. Taken together, these results demonstrate that the inhibitory effects of tetanus toxin on [^{3}H]ACh release are observed only in cultures that are grown for extended periods in the presence of NGF.

At this stage of the project we had esablished that the PC12 cell line was an exceelent model system of the tetanus toxin intoxication pathway. In the next phase of the research program we exploited this PC12 cell system to identify underlying molecular mechanisms in the tetanus toxin action.

It is well recognized that cGMP levels rise in nervous tissue in response to depolarizing stimuli (Nathanson, 1977; Goldberg and Haddox, 1977). We have examined the effects of depolarization on cGMP levels in PC12 cells. As shown in Figure 7, when PC12 cells were stimulated with veratridine, K^+, carbachol, or Ba^{2+}, cGMP levels were increased 7-12 fold.

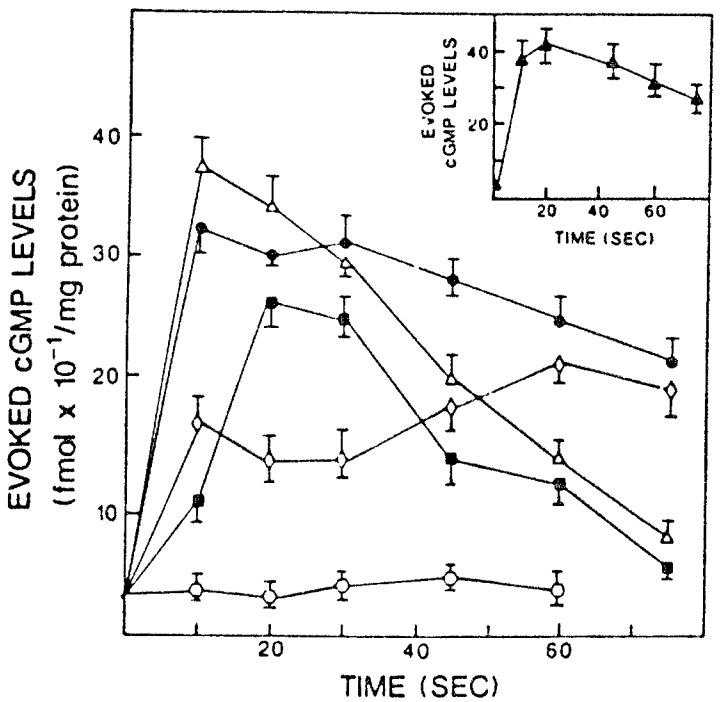


Figure 7. Time Course of stimulus-induced cGMP accumulation in PC12 cells. Cells were cultured in 35 mm dishes with NGF. The experiments were initiated by incubating the attached cells with depolarizing buffers at 37°C. cGMP levels were measured by RIA methods. Shown are the cGMP levels when the cells were exposed to buffer supplemented with 200 μM veratridine (■), 1 mM carbachol (△), 2 mM $BaCl_2$ (●), or 30 mM KCl (△). Inset shows the time course for cGMP levels in cultures that have been treated with carbachol in an identical manner except that PC12 cultures were pretreated for 2 min with 100 μM IBMX.

Time course studies revealed that there was a biphasic response, a rapid increase, followed by a declining phase. This declining phase is most likely due to the activity of phosphodiesterase since the PDE inhibitor, IBMX, attenuated this phase (Fig. 7 inset).

An important discovery was that tetanus toxin blocks the depolarization-induced increases in cGMP. As shown in Table 1, when PC 12 cells were preincubated with 10 nM tetanus toxin for 16 hr, the cGMP response to all of the depolarizing stimuli were inhibited by as much as 80 %.

TABLE I.
Effect of Tetanus Toxin on Depolarization Induced Accumulation of cGMP

Incubation Conditions	Intracellular cGMP level (fmol x 10^{-1}/well)		
	Control	Toxin	% Control
Veratridine	31 ± 1.5	12 ± 0.5	39
Carbachol	37 ± 2.7	13 ± 0.7	35
Barium	74 ± 5.0	15 ± 0.6	20
Potassium	106 ± 7.8	39 ± 3.1	37

The effects of tetanus toxin on cGMP accumulation were studied in more detail. The potency of tetanus toxin and the time course for its effects were characterized. The results are shown in Figs. 8 and 9.

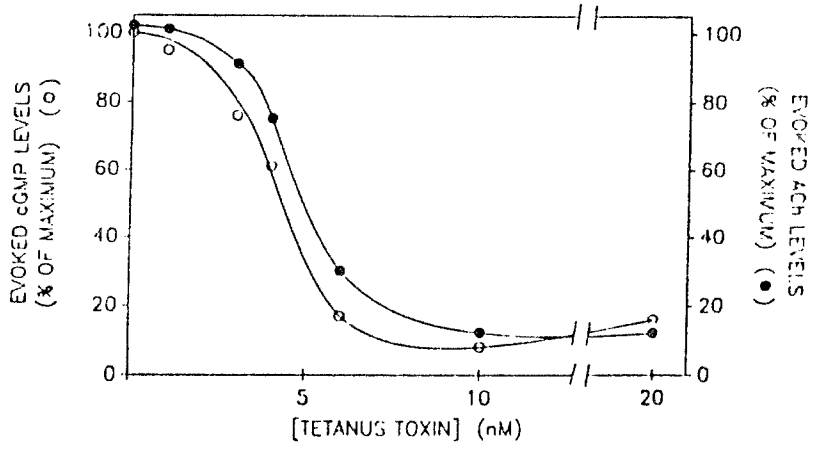


Figure 8. Dose-response curve of tetanus toxin action on K⁺-stimulated [³H]ACh release and cGMP accumulation. PC12 cells were preincubated for 3 hr with increasing doses of tetanus toxin at 37°C. At the end of the incubation period [³H]ACh release and cGMP accumulation in response to stimulation with 30 mM K⁺ were measured from the same culture well. Shown are the release of [³H]ACh (○) and cGMP accumulation (○) after 2 min incubations expressed as percent of the maximal value in control cultures that were not exposed to toxin. These results are the means of 2-3 experiments each performed in sextuplicate.

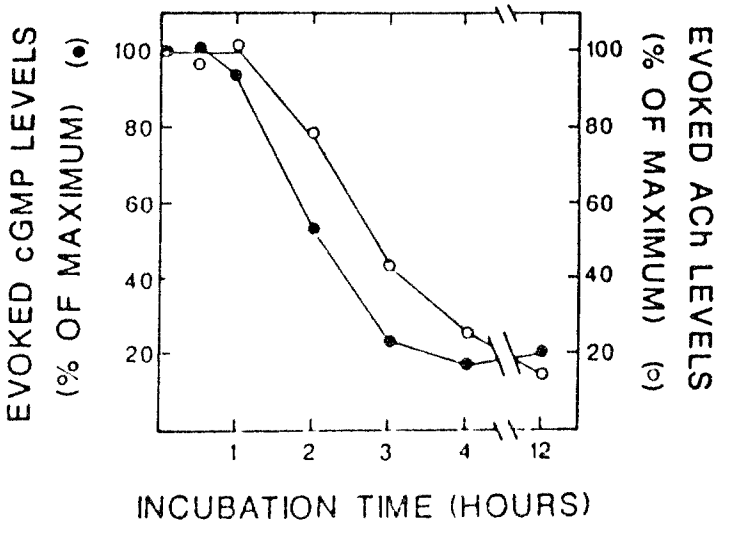


Figure 9. Time course of tetanus toxin action on K⁺-Stimulated [³H]ACh release and cGMP accumulation. [³H]Ch-prelabeled PC12 cells were incubated with 10 nM tetanus toxin at 37°C. At various times the cultures were removed from the incubator and the K⁺-evoked release of [³H]ACh (○) and cGMP accumulation (○) accumulation were measured in the same culture wells.

These data illustrate that there is a very close relation between the potency of toxin in inhibiting

ACh release and cGMP accumulation. Further, there is a nearly identical time course for the development of the two effects evoked by the toxin. Taken together, these results provide strong circumstantial evidence that the toxin-evoked inhibition of cGMP accumulation and ACh release are causally related.

An important finding from our laboratory is that the differentiation state of the PC12 cell cultures was a crucial factor in determining the sensitivity of the cells to tetanus toxin (Sandberg *et al.*1989). In particular, we have found that the cells must be grown cultured in the presence of nerve growth factor (NGF) in order to obtain tetanus-sensitive cultures. Experiments were performed to determine if the tetanus toxin-evoked inhibition of cGMP accumulation was also related to cell differentiation. As shown in Fig. 10, tetanus toxin blocked Ba^{2+}-evoked cGMP accumulation only in cells that had been cultured with NGF.

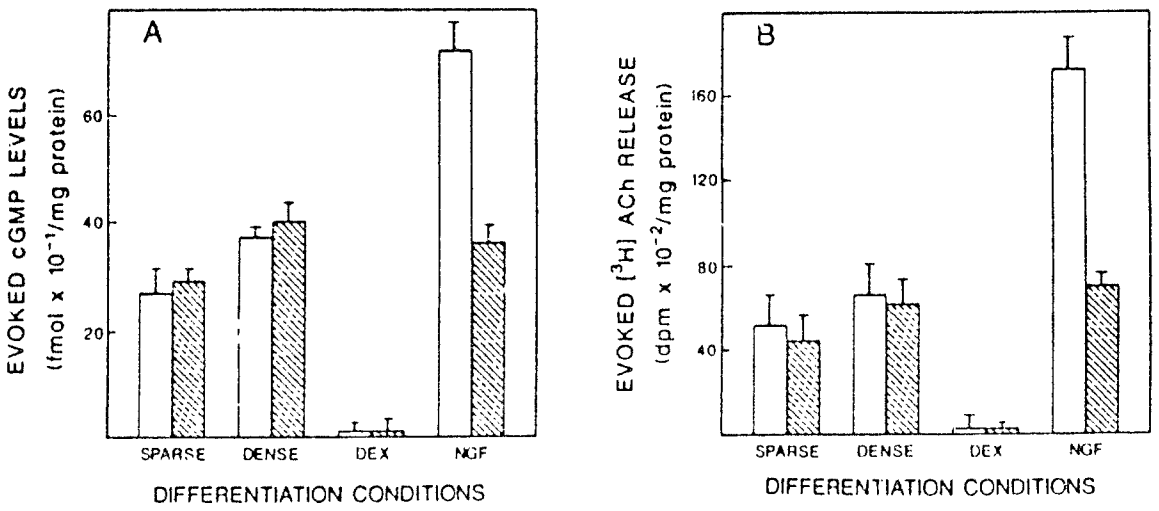


Figure 10. Effect of tetanus toxin on Ca^{2+}-evoked [^{3}H]ACh release and cGMP accumulation from PC12 cells grown under various differentiation conditions. Ba^{2+}-evoked cGMP accumulation (Panel A) or [^{3}H]ACh release (Panel B) were measured. Evoked [^{3}H]ACh release and cGMP accumulation were measured in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM 16-18 h preincubations at 37°C) from PC12 cells grown under a variety of conditions: 14 days at 5 x 10^4 cells/10 cm^2, in the presence of 1 x 10^{-6} M dexamethasone (DEX); 14 days at 5 x 10^4 cells/10 cm^2, in the presence of 100 ng/ml nerve growth factor (NGF); 7 days, at high density (5 x 10^5 cells/ 10 cm^2) (Dense); or at low density (5 x 10^4 cells/ 10 cm^2) (Sparse).

These data show that tetanus' effects on ACh release and cGMP accumulation depend on the differentiation state of PC12 cells in an identical manner. Detailed examination of the development of the toxin sensitivity in NGF-treated cultures revealed that the cells became sensitive to tetanus toxin only after culturing in NGF for at least 8 days. These results are shown in Fig. 5.

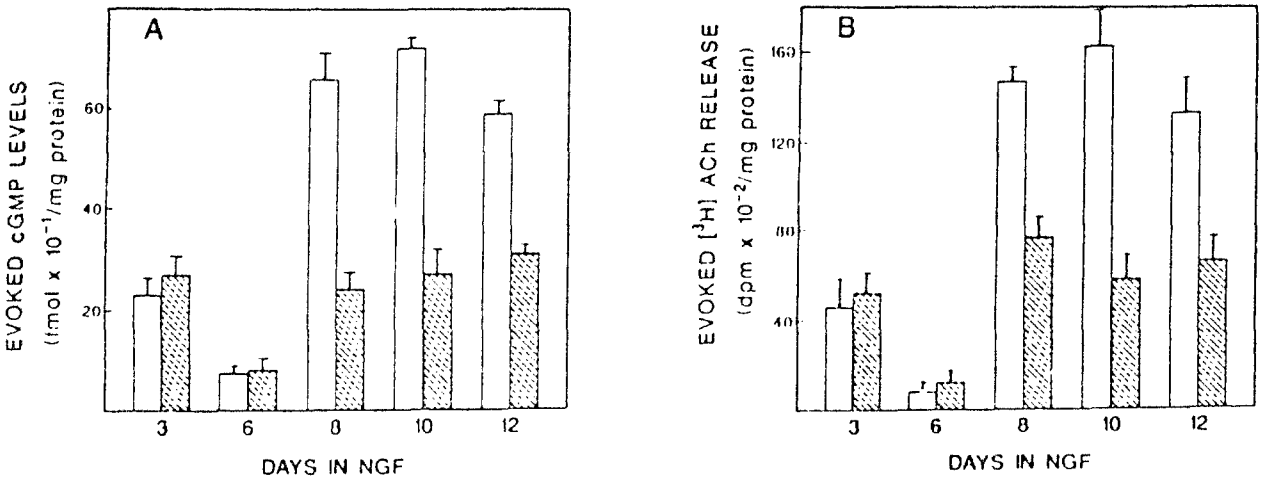


Figure 11. Effect of tetanus toxin on Ba^{2+}-evoked [^{3}H]ACh release and cGMP accumulation from PC12 cells as a function of days in NGF. Evoked [^{3}H]ACh release (Panel B) and cGMP (Panel A) accumulation were measured as a function of culture days in NGF (100 ng/ml) in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM; 16-18 h incubation at 37°C).

In summary, it is clear that the differentiation state of the cells is a crucial factor in determining the sensitivity of the cells to tetanus toxin as assessed either at the biochemical of functional level. The factors responsible for the expression of tetanus toxin sensitivity are intriguing but not known at present.

The next phase of this project was devoted to experiments that would further explore the

mechanism of action of tetanus toxin with a focus on the role of cGMP in the process. In order to achieve this goal we decided to develop methods to permeabilize PC12 cells with a pore-forming exotoxin, α-toxin, obtained from *Staph. aureus.* This toxin has been utilized effectively to examine neurosecretion in several neural preparations (Ahnert-Hilger *et al.*1985; Thelestam and Blomqvist, 1988). The advantage of this approach is that in permeabilized cells one has direct access to the intracellular space to which one can apply probes in a controlled manner. Initial experiments with these cells demonstrated that both dopamine (DA) and acetylcholine (ACh) were secreted from such cells in a Ca^{2+}-dependent manner (Figure 12). The response was biphasic, with half maximal effects observed at 0.6 μM and 20 μM free Ca^{2+}.

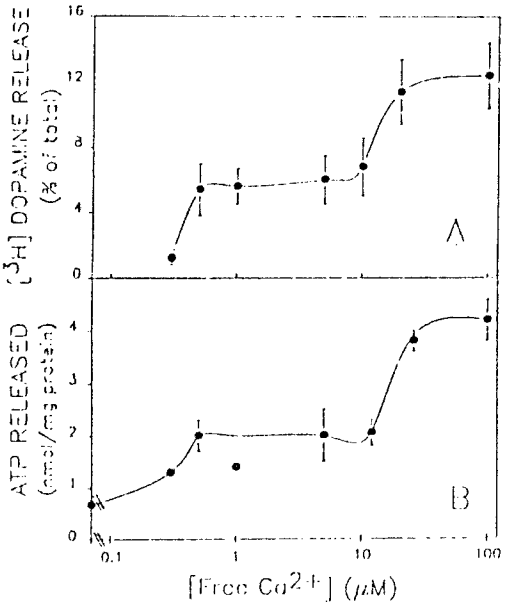


Figure 12. Ca^{2+}-dependent release of [^{3}H]DA and ATP from α-toxin-permeabilized cells. In Panel A, release of [^{3}H]DA was determined from prelabeled PC12 cells. Data are expressed as Ca^{2+}-dependent transmitter release after subtraction of values in the absence of Ca^{2+} (representing 5-12% of total). In panel B, ATP release was monitored in experiments similar to those in Panel A. ATP was determined using a luciferase assay. Ca^{2+}-independent release of ATP was 0.6 nmol/mg protein. Data points represent means ± SEM (n=9, Panel A; n=3, Panel B).

Experiments were performed to determine whether [^{3}H]DA release in both Ca^{2+}-dependent

phases was derived from transmitter stored in vesicles. Release of ATP, a nucleotide which has been shown to be co-localized with neurotransmitters in vesicles (Green and Rein, 1977), was used as an independent index of vesicular release. As shown in Figure 12B, the release of ATP from permeabilized cells showed a biphasic response to Ca^{2+} nearly identical to that for [^{3}H]DA.

As indicated above, there is accumulating evidence from this laboratory that tetanus toxin exhibits its effects by altering a step involved in cGMP metabolism. Such data suggests that cGMP may be an important signalling molecule in regulating neurosecretion in general. As an initial approach to examine this hypothesis, experiments were performed to examine the effects of cGMP on [^{3}H]DA release in permeabilized PC12 cells. As shown in Figure 13, cGMP did evoke the release of DA from such cells in a dose- and Ca^{2+}-dependent manner.

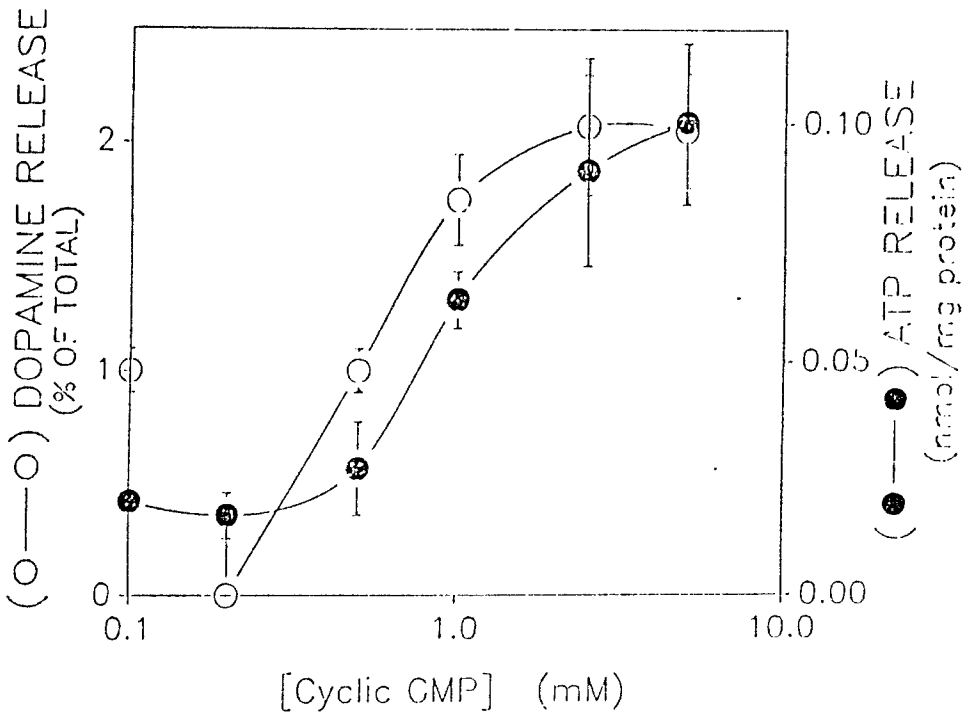


Figure 13. Dose-response curves for cGMP-mediated release of [^{3}H]DA and ATP from permeabilized PC12 cells. Cells were incubated in the presence ( ) or absence ( ) of [^{3}H]DA, washed and exposed to α-toxin (100 units/ml) prior to further incubation for 6 min in the same buffer containing the concentrations of cGMP shown. Release of radiolabel or ATP in the absence of nucleotide was subtracted from experimental values to show the specific increase due to cGMP.

The time course for the cGMP-evoked release of [^{3}H]DA is shown in Figure 14. After the cells were exposed to 1mM cGMP, there was a lag period of 1 min, after which, [^{3}H]DA release occurred, reaching maximal values by 3 min.

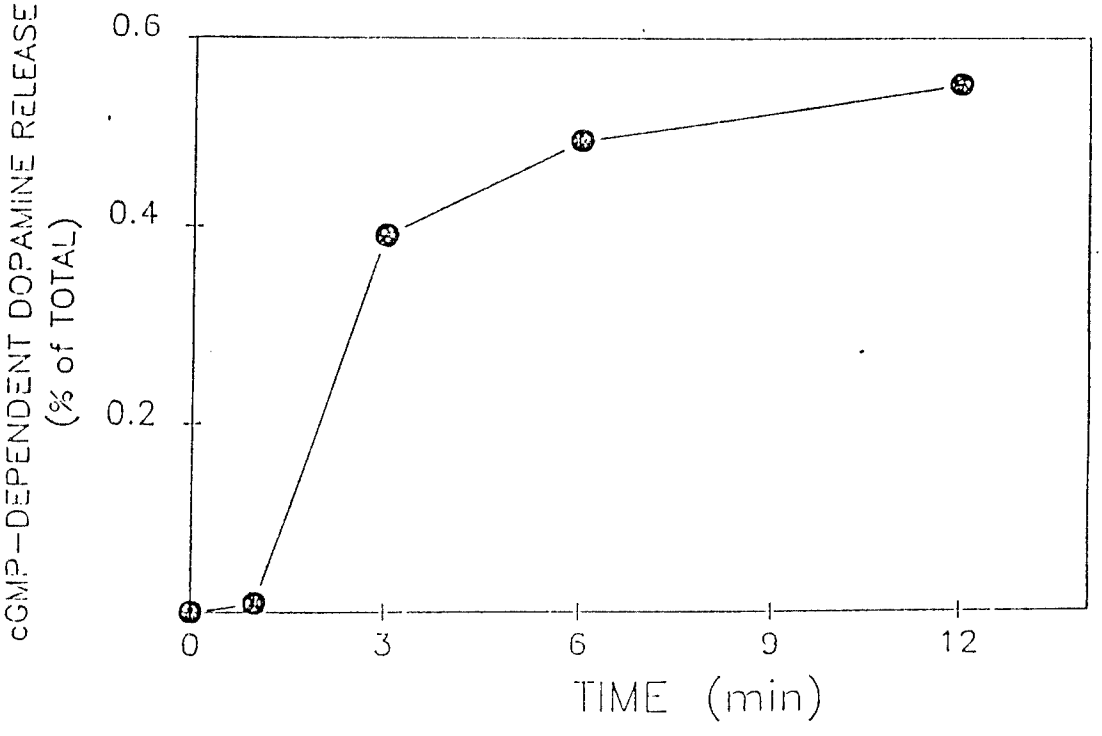


Figure 14. Time course of cGMP-mediated release of [^{3}H]DA. Cells, incubated in the presence of [^{3}H]DA were permeabilized with α-toxin in KG buffer (10mM EGTA). Permeabilization medium was removed and replaced with fresh buffer in the presence or absence of 1mM cGMP. At the times indicated this medium was removed and specific release due to nucleotide was determined at each time point. Values shown are from a single experiment (+/- SEM; n=3). Release in the absence of cGMP represented 2.3% of total label at 3 min.

The nucleotide specificity for evoked release of transmitter in the absence of Ca^{2+} was examined. Only analogues of cGMP were effective in evoking [^{3}H]DA release under the conditions used. In contrast, GMP and other cyclic nucleotides were not active in this system (data not

shown). Thus, taken together, these data suggest that cGMP can play a role in regulating neurosecretion from PC12 cells.

Putative sites of action of cGMP. A possible explanation for the action of cGMP on secretion is that it may release Ca^{2+} from intracellular stores. However, since 10 mM EGTA was used in the release buffer, it seemed unlikely that any released Ca^{2+} would not be buffered and could result in a Ca^{2+} transient sufficient to stimulate secretion. Experiments were performed to confirm this hypothesis. PC12 cells, permeabilized in the absence of Ca^{2+}, were treated with A23187 in order to release Ca^{2+} from intracellular stores. In the absence of EGTA this treatment resulted in increased release of transmitter (Figure 15). However, release observed in the presence of ionophore was reduced to control levels if the concentration of EGTA was greater than 1mM (Figure 15). These results argue against the possibility that cGMP-evoked [^{3}H]DA release, measured in the presence of 10mM EGTA, results from release of a cGMP-sensitive intracellular pool of Ca^{2+}.

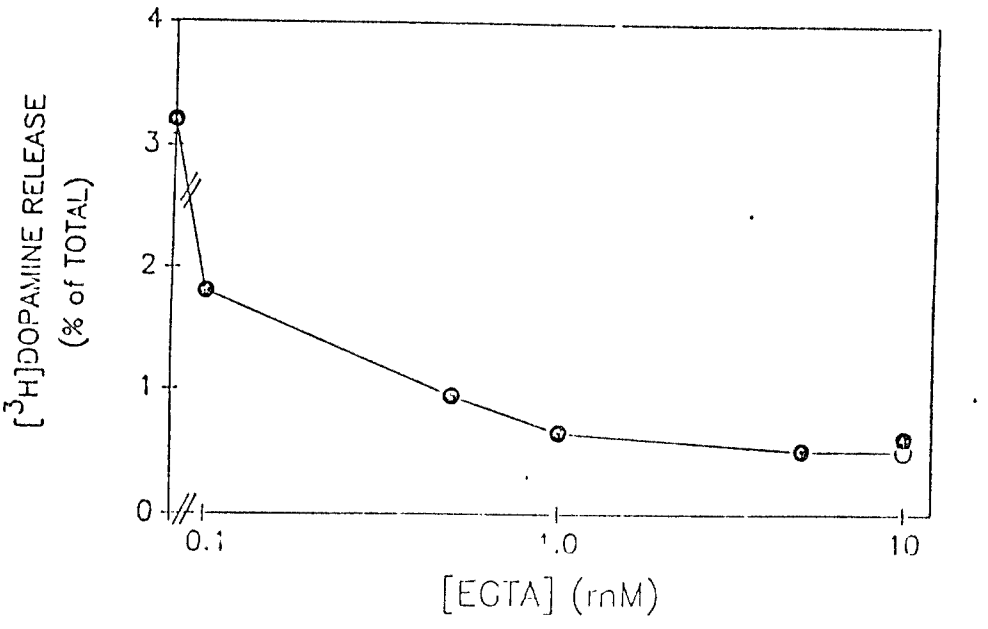


Figure 15. Effects of EGTA concentration on Ca^{2+} ionophore-induced release of [^{3}H]DA. PC12 cells were preincubated with [^{3}H]DA as detailed in the text. Subsequent washes were made in KG buffer (pH 7.4) containing 0-10mM EGTA (Mg^{2+} adjusted to maintain a free concentration of 2.8mM). After the cells were permeabilized with α-toxin (100 units/ml) in the appropriate EGTA buffer, they were incubated for 6 min at 37°C in the presence ( ) or absence ( ) of A23187 (5 μM). Data points represent the mean of triplicate determinations from a single experiment.

The action of cGMP may involve a cGMP-dependent kinase. While the effects of cGMP did not require the presence of exogenous ATP (data not shown), there may be sufficient ATP still present in permeabilized PC12 cells to maintain phosphorylation-mediated events. This hypothesis was supported by results from experiments in which ATP levels were measured in permeabilized and intact cells and found to be 44 and 97 nmol/mg of protein respectively. Thus in order to further explore this hypothesis, the effects of a non-hydrolyzable analogue of ATP on cGMP-evoked [^{3}H]DA release was examined. Addition of AMPPNP (Yount *et al.*1971) completely inhibited any increase in secretion due to cGMP (Figure 16).

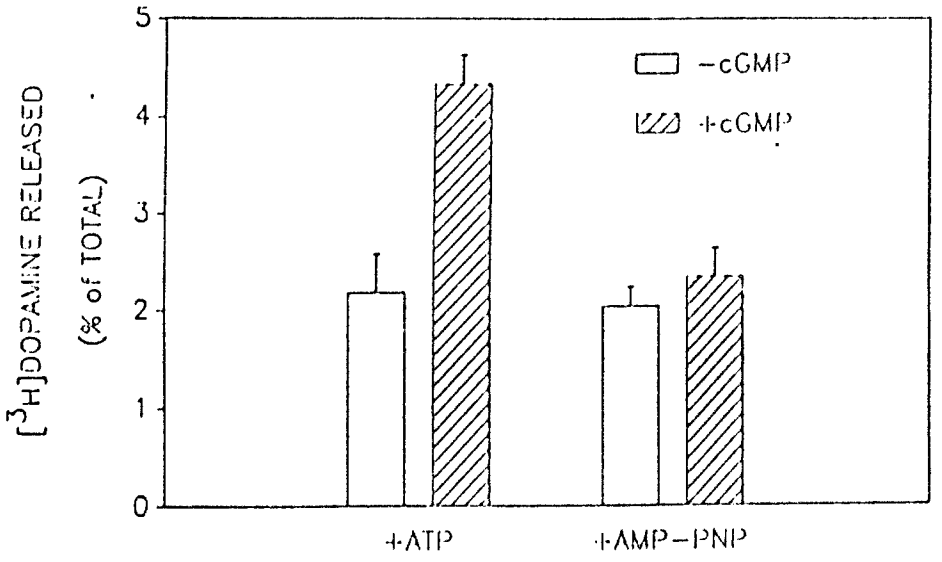


Figure 16. Effects of ATP analogue, AMPPNP, on cGMP-induced [^{3}H]DA release. [^{3}H]DA-prelabeled cells were permeabilized in the presence (shaded bars) or absence (open bars) of 1mM cGMP and specific release of [^{3}H]DA was quantitated. All buffers were supplemented with 1mM ATP (control) or 1mM AMP-PNP.

These results suggest that hydrolysis of ATP is important in mediating the effects of cGMP. Thus, these data suggest the importance of phosphorylation-mediated events, through the activation of a cGMP-activated kinase for example, in the stimulation of secretion by cGMP.

CONCLUSIONS

During the early phase of this project we were successful in establishing a cultured cell model system, the PC12 pheochromocytoma cell line, to study the mechanism of action of tetanus toxin. Further we have studied the characteristics of the intoxication pathway (Sandberg *et al.*1989) and have found that it is analogous to that which has been characterized, to some extent, *in vivo* (Simpson, 1986; Habermann and Dreyer, 1986). Thus, we were very successful in establishing a valid model system with which we could study the molecular mechanisms of action of tetanus toxin. The major thrust during the next phase of the contract was to exploit this well characterized model system to gain insight into the molecular mechanism of action of tetanus toxin. The major conclusions from this work are: (i) tetanus toxin inhibits stimulus-evoked cGMP levels in PC12 cells under conditions in which it blocks stimulus-evoked ACh release; (ii) the inhibitory effects of tetanus on ACh release are rapidly reversed with cGMP analogs; and (iii) a cGMP specific phosphodiesterase is a possible site of action for tetanus toxin since phosphodiesterase inhibitors restored stimulus-evoked ACh release and cGMP levels in a similar manner.

Results obtained during this contract identify the metabolic pathway for cGMP as a potential site of action of tetanus toxin. Preliminary studies have revealed that guanylate cyclase activity is not inhibited in intoxicated PC12 cells. Although we can not rigorously rule out a possible role for this enzyme in toxin action, all of the evidence reported here is consistent with the view that the degradation of cGMP is stimulated in toxin-treated cells. The phosphodiesterase inhibitors, IBMX and zaprinast, were effective in reversing the effects of tetanus toxin on both the inhibition of evoked cGMP accumulation and ACh release in a similar manner. IBMX, a wide spectrum, rather low affinity, phosphodiesterase inhibitor (Weishaar *et al.*1985), partially restored

cGMP levels and ACh release. Zaprinast has been reported to be specific for cGMP-degrading phosphodiesterases in a number of diverse tissues (Weishaar et al., 1985; Luginer et al., 1986; Windquist et al., 1984). Results reported here reveal that this agent was very effective in elevating cGMP levels in control PC12 cells as well. This compound completely restored the stimulus-evoked cGMP response and ACh release after it was applied for 15 min to intoxicated cells. While it is possible that the hydrophobic agents, 8Br-cGMP and zaprinast, act through nonspecific mechanisms, the observation that the effects of tetanus can be reversed by these two distinctly different chemicals that share the common property of elevating cGMP levels in PC12 cells strongly argues against nonspecific mechanisms.

In order to study the mechansisms of acxtion of tetanus in the latter phase we have utilized a preparation of permeabilized, NGF-differentiated, PC12 cells to examine the role of cGMP in neurotransmitter release. An important finding is that cGMP can stimulate neurotransmitter release from such cells in a Ca^{2+}-independent manner. Further, NGF-differentiated PC12 cells show two phases of vesicular neurotransmitter release that can be distinguished not only by their differential sensitivity to Ca^{2+}, but also in their sensitivity to cGMP.

Permeabilized NGF-treated PC12 cells retain their ability to release catecholamines in response to Ca^{2+}. The Ca^{2+} dose-response curve for release of catecholamines revealed two phases of neurotransmitter release which is similar to that reported for non-differentiated PC12 cells (Ahnert-Hilger *et al.*1985). Two series of experiments indicated that both the high and low affinity Ca^{2+}-dependent release originated from a vesicular pool(s); firstly, preincubation with the plant alkaloid reserpine, which significantly reduces the level of transmitter within vesicles (Kittner *et al.*1987), inhibited Ca^{2+}-dependent secretion from both phases. Second, the release of ATP, which is stored in secretory vesicles with transmitter and co-released upon stimulation (Green and Rein, 1977), exhibits a similar biphasic response to Ca^{2+}. Thus, although the biological

significance of these two phases of transmitter release remain to be defined, they arise from pools of secretory vesicles.

An important goal of the present study was to verify the hypothesis, presented elsewhere (Sandberg *et al.*1989), that cGMP may play a role as a signalling molecule in secretion. Several results presented here support the conclusion that cGMP is involved in this process; under nominally Ca^{2+}-free conditions (pCa>9), cGMP stimulates transmitter release in a time-dependent manner; the co-release of ATP indicates that cGMP-evoked release of DA was derived from vesicular pools; the magnitude of cGMP-evoked release in Ca^{2+}-free medium is similar to that evoked by excitatory concentrations of free Ca^{2+} (1-10μM). However, while the effects of cGMP were dose-dependent and highly specific (i.e. DA release was seen only for cyclic analogues of guanine nucleotides), it is not clear whether the nucleotide stimulates secretion from the same population of vesicles as Ca^{2+}.

Dose-response studies revealed that, under the conditions used, half maximal doses of cGMP were in the range of 500μM. These levels may be higher than expected in a physiological context. However, several results indicate that the apparent potency of cGMP is reduced due to two factors; a lack of complete permeability of the plasma membrane to cGMP and degradation of the nucleotide. Permeabilization of cells with saponin (which produces larger pores than α-toxin (Ahnert-Hilger and Gratzl, 1988)) increased the potency of cGMP by 40%. Furthermore, when cells were incubated with [^{3}H]cGMP, 60% of the cell-associated nucleotide was degraded within 3 min. Inclusion of phosphodiesterase inhibitors partially reversed this degradation and increased the apparent potency of cGMP. Thus, while it is difficult to accurately estimate the effective concentration of intracellular cGMP in these experiments, it is clear that cGMP is significantly more potent than estimated by the half-maximal concentration of the dose-response relation.

While many of the experiments were performed in the absence of Ca^{2+}, under physiological conditions Ca^{2+} would be present. Therefore it was important to determine if there were interactions between Ca^{2+} and cGMP on secretion. Transmitter release induced by cGMP was observed only in Ca^{2+}-free buffers. While a small increase in release was observed if cGMP was present during incubations with low Ca^{2+} concentrations (<1μM; data not shown), cGMP effects were not additive with release induced by 10μM free Ca^{2+}. Although this effect could be explained by a cGMP-mediated release of Ca^{2+} from intracellular stores this is unlikely, since, in the presence of 10mM EGTA, intracellular Ca^{2+} levels are effectively buffered. Furthermore, in contrast to its stimulatory action under conditions of low free Ca^{2+}, cGMP was actually inhibitory to release induced by 100μM free Ca^{2+}. It was also clear that AMPPNP inhibited the action of cGMP suggesting that hydrolyzable ATP is required for the action of the nucleotide. These results suggest that a cGMP-dependent kinase may be an important mediator of the response. Further experiments are needed to clarify this issue.

Thus, in summary, cGMP was found to stimulate the rapid release of neurotransmitter from permeabilized PC12 cells under essentially Ca^{2+}-free conditions. Further, in the presence of Ca^{2+}, cGMP regulated one phase or mode of Ca^{2+}-dependent release. These observations provide new insight on the importance of cGMP in regulating the molecular events that are triggered by depolarization and that lead to neurotransmitter release. It will be important in future studies to examine the effects of tetanus and botulinum toxins on the process.

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2. Andrea Grandin, MS -- Research Assistant, 100% time
3. David Evans, PhD -- Research Associate, 100% time
4. Shirley Gaa, BS - Research Assistant, 25% time

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